2829-Pos Board B599

miR-208A Targeted Suppression of PDE4D Directly Enhances Myocyte Contractile Function via PKA-Mediated Phosphorylation of cTnI and PLN

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Molecular inotropy refers to cardiac myocyte contractile status that can be titrated, positively or negatively, to affect overall heart pump performance. Although inotropic drugs have been in clinical practice for many decades, there is an urgent need to discover new inotropes with unique mechanisms of action for the treatment of heart failure. Here, we investigate the prospect of micro-RNAs to directly modify downstream inotropic signaling pathways for improving contractile function. We focused on miR-208a owing in part to its known restricted expression profile in the myocardium. Results show that acute miR-208a expression, at 4-5 fold over endogenous miR-208a and independent of altered host myosin gene expression, confers significant positive inotropy and faster relaxation compared to miR-208a-mutant and untreated control adult cardiac myocytes ($P<0.05$). Using in vitro cardiac stress testing, miR-208a amplified the inotropic and relaxation responses to increased stimulation frequency. MiR-208a also promoted fast calcium transient decay with no change in peak calcium accounting in part for enhanced relaxation. To gain insight into mechanism, we analyzed in silico putative miR-208a targets, focusing on potential inotropic signaling targets. Interestingly, we provide evidence that miR-208a has a direct effect to negatively regulate expression of PDE4D, but does not affect PDE5A in myocytes. Consistent with these findings, phosphorylation of cTnI and PLN at PKA sites was increased in myocytes after acute miR-208a expression. Taken together, we show for the first time that miR-208a confers positive inotropy and enhances relaxation in myocytes by PKA mediated phosphorylation of cTnI and PLN through a mechanism of direct suppression of PDE4D. Because heart failure is associated with decreased phosphorylation of cTnI and PLN, miR-208a may represent a new therapeutic modality for enhancing ventricular myocyte performance via the PDE4D-cAMP-PKA signaling pathway.

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Regulatory Light Chain Phosphorylation Mimic S15D Causes Partial Rescue of Isometric Force Production in FHC Causing Mutation D166V

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Familial hypertrophic cardiomyopathy (FHC) is characterized by a pathological thickening of the muscle surrounding the heart and is the leading cause for sudden cardiac death in young people. There are numerous mutations in sarcomeric proteins that have been implicated in causing FHC. Myosin, the molecular motor that powers cardiac muscle contraction, consists of a globular domain and an elongated α-helical neck region, which is thought to undergo large conformational changes during muscle contraction. The myosin regulatory light chain (RLC) functions to support the neck region, therefore it is not surprising that several single amino acid substitutions in the RLC have been implicated in FHC.

Here we studied D166V, an RLC point mutation that is associated with increased left ventricular wall thickness, abnormal electrocardiogram, and decreased isometric force and ATPase in skinned fibers from transgenic mouse hearts. We have expressed porcine cardiac β myosin (the same isoform found in humans) and replaced the endogenous RLC with a human RLC. The exchanged RLC contained either wild type (WT), D166V, S15A, or a double mutant D166V/S15A, or D166V/S15D. The S15D mutation served as a phosphorylation mimic. We performed frictional loading assays using a modified *in vitro* motility assay and determined the average force produced by a bed of monomeric myosin. While maximal unloaded velocity was unchanged for all of the mutants studied, the force of D166V and D166V/S15A was significantly reduced compared to WT, with an additive decrease in the double mutant. D166V/S15D resulted in a significant increase in force compared to D166V/S15A and was restored to near WT levels. These results suggest that D166V causes a reduction in myosin isometric force production and phosphorylation may act to recover it.

2831-Pos Board B601

Physiological Effects of FHC-Causing K104E Mutation in the Myosin Regulatory Light Chain

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We have studied the physiological effects of the Lysine 104 to Glutamic Acid (K104E) mutation in the ventricular myosin regulatory light chain (RLC), shown to cause familial hypertrophic cardiomyopathy (FHC). *In vitro* and *in vivo* experiments were performed using transgenic (Tg) mouse cardiac muscle preparations carrying the K104E-RLC mutation. We observed a slight but significant decrease in maximal force ($\Delta F_{max} \cong 8 kN/m^2$) and an increase in the Ca^{2+}-sensitivity ($\Delta pCa_{50} \cong 0.1$) of isometric contraction in glycerinated skinned muscle fibers from Tg-K104E compared to Tg-WT mice. No mutant related changes in rigor binding of transgenic K104E mouse myosin to pyrene-actin were observed. Likewise, no changes in actomyosin or myofibrillar ATPase activities were monitored. Histological examination of Masson's trichrome stained Tg-hearts showed signs of fibrosis in 8 mo-old Tg-K104E mice compared to age matched Tg-WT. These observed changes in myocyte organization in Tg-K104E mice could be due to ~4-fold decrease in the myosin heavy chain-RLC interaction determined by the binding of bacterially expressed K104E mutant to RLC-depleted porcine myosin. Echocardiography examination of senescent Tg-K104E mice confirmed a hypetrophic phenotype and showed a significantly enlarged interventricular septum and LVPWd (left ventricular posterior wall in diasole), ~1.6-fold increase in LV mass and significantly decreased LVIDs (LV inner diameter in systole) compared to Tg-WT mice. However, EF (ejection fraction) was higher in Tg-K104E mice ($73 \pm 8\%$) compared to $61 \pm 7\%$ observed in Tg-WT mice. In addition, Doppler E velocity was also 1.3-fold higher in Tg-K104E mice compared to Tg-WT. These results confirm a mutation induced hypertrophic phenotype in Tg-K104E mice, similar to the patients carrying this FHC-mutation. No drastic changes at the level of actomyosin interaction or in cardiac function assessed in Tg-K104E mice suggest that the mutation might be associated with good prognosis. Supported by NIH-HL071778 (DSC).

2832-Pos Board B602

Myosin Regulatory Light Chain Phosphorylation Rescues Cardiac Dysfunction Caused by Familial Hypertrophic Cardiomyopathy-Linked Mutations

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In this report, we compared the role of cardiac myosin regulatory light chain (RLC) phosphorylation on cardiac function in skinned muscle preparations containing two RLC mutations, R58Q (arginine to glutamine) and D166V (aspartic acid to valine), both linked with a malignant disease phenotype. Previous studies on D166V-transgenic mice showed that the myosin light chain kinase (MLCK)-induced phosphorylation of D166V mouse myocardium was able to alleviate detrimental functional effects caused by this mutation. In this study, we used recombinant phosphomimetic S15D (serine to aspartate) mutation in the D166V background exchanged into porcine cardiac myosin and investigated the effect of constitutively phosphorylated RLC proteins on the actomyosin interaction. The actin-activated myosin ATPase activity, which was decreased in D166V- exchanged myosin, was partially rescued in S15D-D166V exchanged myosin reaching the level observed for WT-reconstituted myosin. Similarly, myosin reconstituted with S15D-D166V mutant showed an increase in the binding to fluorescently labeled actin compared to D166V-reconstituted myosin, with Kd=1.9 μM and Kd=41 μM, respectively. To further investigate whether MLCK-phosphorylation could rescue the phenotype associated with the R58Q mutation, we studied force development in transgenic R58Q-mouse papillary muscle fibers. Compared to Tg-WT, a drastic reduction in maximal force and myofibrillar ATPase activity was observed in samples carrying the R58Q mutation. However, MLCK induced phosphorylation of R58Q muscle fibers resulted in significantly increased maximal force and myofibrillar ATPase activity. These results suggest that RLC phosphorylation plays an important role not only in the physiological performance of the heart, but also helps to maintain normal cardiac function in the diseased myocardium. Our findings may contribute to the development of targeted cellular therapeutic approaches to limit FHC related cardiac dysfunction. Supported by AHA-10POST3420009 (PM) and NIH- HL071778 and HL090786 (DSC).

2833-Pos Board B603

Novel Phosphorylation Sites and Reduced Phosphorylation Levels in Human Cardiac MyBP-C from Failing Hearts

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Cardiac myosin binding protein-C (cMyBP-C) is a large multidomain protein associated with the thick filaments. Phosphorylation of cMyBP-C is a regulator of cardiac contractility and it is known that the phosphorylation status of

cMyBP-C is altered during heart failure (HF). There are several identified phosphorylated residues, including the regulatory phosphorylation sites Ser275, Ser284 and Ser304. Methods: Comparison of the extent of MyBP-C phosphorylation status in failing (N=10) and non-failing (N=10) human left ventricle (LV) tissue. The myofilament subproteome was extracted using the 'IN Sequence' method prior to analysis with some samples dephosphorylated using alkaline phosphatase as control. Sample analysis was carried out using gel-(e.g. phos-tag) and mass spectrometry (MS)-based methods. Results: Using an MS-based phospho-peptide ($Ti0_2$ chromatography) enrichment strategy we identified novel phosphorylation sites at residues Ser286 and Thr290, located in the M-motif. These sites were as well observed in MyBP-C from LV canine hearts. A novel site at residue Thr1109, the titin binding domain C9, was only observed in the samples without phospho-enrichment. Our results indicate a marked reduction in cMyBP-C phosphorylation at residues Ser284, Ser286, Thr290 and Thr1109 in end-stage HF compared to the non-failing group. Conclusion: This study provides evidence for novel phosphorylation sites on cMyBP-C both in human and canine hearts and reduced phosphorylation levels in the end-stage failing heart.

2834-Pos Board B604

Effects of HCM Missense Mutations in the M Domain of Cardiac Myosin Binding Protein C on Calcium Sensitivity of Force and Rate in Rat Trabeculae

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Recombinant N-terminal domains of cardiac myosin binding protein C (cMyBP-C) increase calcium-sensitivity of force and the rate of tension redevelopement (ktr) when added to permeabilized rat trabeculae. We previously demonstrated that the regulatory domain of cMyBP-C, referred to as the MyBP-C motif (or ''M domain''), is required for these effects. Here we investigated the effects of single amino acid missense substitutions within the M-domain that are associated with human hypertrophic cardiomyopathy (HCM) on force development and ktr in permeabilized trabeculae from rat right ventricles. Individual substitutions (R322Q, E330K, V338D, and L348P) were introduced into a recombinant mouse C1C2 protein (encompassing domains C1-M-C2 of murine cMyBP-C) by site directed mutagenesis and effects were compared to wild-type C1C2. All four of the mutations affected the ability of C1C2 to augment force. Whereas 5 μM wild-type C1C2 induced a pronounced leftward shift in Ca2+ sensitivity of tension (~0.5 pCa units) and increased ktr at all submaximal Ca2+ concentrations, 3 of the mutations reduced the effects of C1C2. Another variant increased the efficacy of C1C2 and increased passive force independent of Ca2+, but reduced maximal Ca2+ activated force. Together these results indicate that cMyBP-C variants associated with HCM could directly disrupt sarcomere contractile properties through gain or loss of functional effects and that at least some cMyBP-C missense mutations may cause disease through a poison polypeptide mechanism. This work is supported by NIH HL080367 to SPH and a DOD NDSEG graduate fellowship to KLB.

2835-Pos Board B605

Common Mechanical Properties of Recombinant and Native Cardiac Myosin Binding Protein-C by Atomic Force Microscope

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Cardiac myosin binding protein-C (cMyBP-C) is a member of the immunoglobulin (Ig) superfamily of proteins and consists of 8 Ig- and 3 fibronectin (Fn)-like domains along with a unique regulatory sequence referred to as the MyBP-C ''motif'' or M-domain. Previously we used atomic force microscopy (AFM) to investigate the mechanical properties of the different domains of murine cMyBP-C expressed using a baculovirus/insect cell expression system. To investigate whether the mechanical properties of cMyBP-C are conserved across species, here we used AFM to investigate the mechanical properties of human recombinant cMyBP-C expressed using a baculovirus/insect cell expression system and native cMyBP-C purified from bovine heart. AFM force-extension spectra were obtained from cMyBP-C molecules by randomly adhering individual molecules to the tip of an AFM cantilever and moving the cantilever to impose a load that stretched the molecules. Results show that the spectra for the human recombinant and bovine native proteins are remarkably similar with the first Ig/Fn-like domain unfolding events occurring at low (~50 pN) forces and the highest stability domains unfolding at ~190 pN. Experiments also revealed frequent unfolding events that appeared coupled such that lower stability domains would often unfold after higher stability domains. These unexpected force ''drops'' were highly reproducible and occurred in spectra from both human cMyBP-C and bovine cMyBP-C. In addition, both recombinant and native cMyBP-C exhibited an ~100 nm long extensible region that could be stretched with less than 50 pN force prior to the unfolding of Ig and Fn-like domains. Combined with our previous results from mouse cMyBP-C, these results establish common mechanical features of cMyBP-C across species. Supported by NIH HL080367.

2836-Pos Board B606

The Effects of C-Terminal Mutations on the Folding of Cardiac Myosin Binding Protein-C

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Cardiac myosin binding protein-C (cMyBP-C) is a modular protein involved in stabilizing interactions with the thick filament of the sarcomere. The N-terminus of cMyBP-C associates with actin and myosin S2 and the C-terminus interacts with titin and myosin rods. While no high-resolution structure of C-terminal cMyBP-C exists, disruption of this region is proposed to destabilize cMyBP-C and adversely affect cardiac structure and function. In particular, deletion of 25 base pairs (Δ25) in the gene encoding for cMyBP-C results in amino acid substitutions in the C10 domain of cMyBP-C (C10 Δ25) which may be associated with the development of hypertrophic and dilated cardiomyopathies by unknown molecular mechanisms. The prevalence of this mutation is approximately 1% of the world population, underscoring the necessity of determining its role(s) in the pathogenesis of cardiomyopathies. In this study, circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopies have been used to examine the conformation of wild-type (Wt) and mutant C-terminal domains of cMyBP-C. Comparison of near UV CD spectra revealed alterations in the packing of aromatic residues in C10 Δ25 suggesting it is less stably folded as compared to C10 Wt. C10 Δ25 exhibited less beta-sheet content than C10 Wt as evidenced by the estimation of secondary structure from CD data. NMR analyses of amide proton/nitrogen chemical shifts and linewidths were used to probe the conformation of C10 domains and to map residues of importance in protein-protein association onto cMyBP-C models. Taken together, these data suggest that the Δ25 mutation structurally modulates cMyBP-C sites involved in binding titin and myosin.

2837-Pos Board B607

Human Signaling Scaffold Protein (mAKAP) Binding Kinetics to PKA and Phosphodiesterase (PDE4DE): Implications for a Possible Role in Heart Failure

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Heart failure is a leading cause of morbidity and mortality in the USA. There are several therapeutic agents available for heart failure management. In particular, agents that block beta-adrenergic receptor improve mortality rate among heart failure patients by enhancing cardiac function. Beta-adrenergic receptor stimulation signals through protein kinase A (PKA) dependent phosphorylation, partly by binding to A-kinase anchoring proteins, influencing calcium homeostasis. In particular, mAKAP (muscle-selective A-kinase anchoring protein) is targeted to specific intracellular compartments resulting in localization of PKA with its substrates as well as to bind with ryanodine receptors and phosphodiesterase-4D3 (PDE4DE). The signal transduction complex formed by the scaffold protein mAKAP at the perinuclear envelop of striated myocytes contains cAMP specific binding protein PDE4D3 which is responsible for cAMP signaling termination. Agents that modify PKA signaling would be expected to mediate an altered inotropic response. From different genomic databases, we have recently identified fifteen human mAKAP coding non-synonymous polymorphisms located within or near key protein binding sites critical to beta-adrenergic receptors signaling. Seven of these mutants were cloned for the purpose of comparing whether those substitution disrupt mAKAP binding to either the PKA binding domain R2alpha or the phosphodiesterase PDE4DE. Using surface plasmon resonance (Biacore 2000) we demonstrate specific binding of wild type mAKAP to PDE4DE. Experiments were run in triplicate and as twofold serial dilutions to explore the kinetics of the interaction and analyzed using Scrubber2 with a 1:1 Langmuir model. Comparative analysis of the binding responses of mutations to mAKAP could provide important information about how these mutations modulate signaling.

2838-Pos Board B608

Single Molecule Studies of a Titin Mutation Linked to Cardiac Disease

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Recently, a mutation in the 10th immunoglobulin(Ig)-like domain in titin's elastic I-band was found in a family affected with arrhythmogenic